

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B7

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/52, 47/30		A1	(11) International Publication Number: WO 98/32427
			(43) International Publication Date: 30 July 1998 (30.07.98)
(21) International Application Number: PCT/US98/01556		Court, Birmingham, AL 35244 (US). ROBERTS, F., Donald [US/US]; 2 Bridge Path Circle, Dover, MA 02030 (US). FRIDEN, Phil [US/US]; 32 Washington Street, Bedford, MA 01730 (US). (74) Agent: BELLAMY, Werten, F., W.; Intellectual Property Law Division, Office of The Judge Advocate General, DA, Suite 713, 901 North Stuart Street, Arlington, VA 22203-1837 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 27 January 1998 (27.01.98)			
(30) Priority Data: 08/789,734 27 January 1997 (27.01.97) US			
(71) Applicant (for all designated States except US): UNITED STATES GOVERNMENT as represented by THE SECRETARY OF THE ARMY [US/US]; Intellectual Property Law Division, OTJAG, DA, Suite 713, 901 North Stuart Street, Arlington, VA 22203-1837 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): SETTERSTROM, Jean, A. [US/US]; 700 Hampton Trace Lane, Alpharetta, GA 30201 (US). VAN HAMONT, John, E. [US/US]; 160A Gardiner Loop, West Point, NY 10996 (US). REID, Robert, H. [US/US]; 10807 McComas Court, Kensington, MD 20895 (US). JACOB, Elliot [US/US]; 11529 Dafford Lane, Silver Spring, MD 20902 (US). JEYANTHI, Ramasubbu [US/US]; 9725 Clocktower Lane #301, Columbia, MD 21046 (US). BOEDEKER, Edgar, C. [US/US]; 7505 Bybrook Lane, Chevy Chase, MD 20815 (US). MCQUEEN, Charles, E. [US/US]; 16805 Ethelwood Terrace, Olney, MD 20832 (US). TICE, Thomas, R. [US/US]; 1915 Forest River		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(54) Title: THERAPEUTIC TREATMENT AND PREVENTION OF INFECTIONS WITH A BIOACTIVE MATERIAL ENCAPSULATED WITHIN A BIODEGRADABLE-BIOCOMPATIBLE POLYMERIC MATRIX			
(57) Abstract			
<p>Novel burst-free, sustained release biocompatible and biodegradable microcapsules which can be programmed to release their active core for variable durations ranging from 1-100 days in an aqueous physiological environment. The microcapsules are comprised of a core of polypeptide or other biologically active agent encapsulated in a matrix of poly(lactide/glycolide) copolymer, which may contain a pharmaceutically acceptable adjuvant, as a blend of uncapped free carboxyl end group and end-capped forms ranging in ratios from 100/0 to 1/99.</p>			

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

-1-

1 THERAPEUTIC TREATMENT AND
2 PREVENTION OF INFECTIONS WITH A BIOACTIVE MATERIALS
3 ENCAPSULATED WITHIN A BIODEGRADABLE-BIOCOMPATIBLE
4 POLYMERIC MATRIX

5 I. GOVERNMENT INTEREST

6 The invention described herein may be manufactured, used and licensed
7 by or for the Government for Governmental purposes without the payment to
8 use of any royalties thereon.

9 II. CROSS REFERENCE

10 This application is a continuation-in-part of U.S. Patent Application
11 Serial No. 08/590,973 filed January 24, 1996 which in turn is a
12 continuation-in-part of U.S. Patent Application Serial No. 08/446,149 filed
13 May 22, 1995, which in turn is a continuation of U.S. Patent Application Serial
14 No. 590,308 dated March 16, 1984.

15 Additionally, this application is a continuation-in-part of U.S. Patent
16 Application Serial No. 08/446,148 filed May 22, 1995, which in turn is a
17 continuation-in-part of U.S. Patent Application Serial No. 08/867,301 filed
18 April 10, 1992 now U.S. Patent No. 5,417,986 issued May 23, 1995, which in
19 turn is a continuation-in-part of U.S. Patent Application Serial No. 590,308
20 filed March 16, 1984.

III. FIELD OF THE INVENTION

-2-

1 This invention relates to compositions comprising active core
2 material(s) such as biologically active agent(s), drug(s) or substance(s)
3 encapsulated within an end-capped or a blend of uncapped and end-capped
4 biodegradable-biocompatible poly(lactide/glycolide) polymeric matrix useful
5 for the effective prevention or treatment of bacterial, viral, fungal, or parasitic
6 infections, and combinations thereof. In the areas of general and orthopedic
7 surgery, and the treatment of patients with infectious or chronic disease
8 conditions, this invention will be especially useful to physicians, dentists and
9 veterinarians.

10 IV. BACKGROUND OF THE INVENTION

11 Wounds characterized by the presence of infection, devitalized tissue,
12 and foreign-body contaminants have high infection rates and are difficult to
13 treat.

14 To prevent infection, in bone and soft tissue systemic antibiotics must
15 be administered within 4 hours after wounding when circulation is optimal.
16 This has been discussed by J.F. Burke in the article entitled "The Effective
17 Period of Preventive Antibiotic Action in Experimental Incisions and Dermal
18 Lesions", Surgery, Vol. 50, Page 161 (1961). If treatment of bacterial
19 infections is delayed, a milieu for bacterial growth develops which results in
20 complications associated with established infections. (G. Rodeheaver et al.,
21 "Proteolytic Enzymes as Adjuncts to Antibiotic Prophylaxis of Surgical
22 Wounds", American Journal of Surgery, Vol. 127, Page 564 (1974)). Once
23 infections are established it becomes difficult to systemically administer certain
24 antibiotics for extended periods at levels that are safe and effective at the

-3-

1 wound site. Unless administered locally, drugs are distributed throughout the
2 body, and the amount of drug hitting its target is only a small part of the total
3 dose. This ineffective use of the drug is compounded in the trauma patient by
4 hypovolemic shock, which results in a decreased vascular flow to tissues. (L.
5 E. Gelin et al., "Trauma Workshop Report: Shock rheology and Oxygen
6 Transport", Journal Trauma, Vol. 10, Page 1078 (1970)).

7 Additionally, infections caused by multiple-antibiotic resistant bacterial
8 are on the up-swing and we are on the verge of a potential world-wide medical
9 disaster. According to the Centers for Disease Control, 13,300 patients died
10 in U.S. hospitals in 1992 from infections caused by antibiotic-resistant
11 bacteria. Methicillin-resistant *S. aureus* (MRSA) is rapidly emerging as the
12 "pathogen of the 90's":

13 a. Some major teaching hospitals in U.S. report that up to 40%
14 of strains of *S. aureus* isolated from patients are resistant to methicillin. Many
15 of these MRSA strains are susceptible only to a single antibiotic (vancomycin).

16 b. Should MRSA also develop resistance to vancomycin, the
17 mortality rate among patients who develop MRSA infections could approach
18 80%, thereby increasing the threat of this infectious killer.

19 Moreover, Vancomycin resistance is on the up-swing:

20 a. 20% of Enterococci are now resistant to vancomycin

21 b. In 1989, only one hospital in New York City reported
22 vancomycin-resistant Enterococci. By 1991, the number of hospitals reporting
23 vancomycin resistance rose to 38.

1 c. transfer of vancomycin-resistant gene (via plasmid) has been
2 shown experimentally between Enterococcus and S. aureus.

3 Many major pharmaceutical companies around the world have either
4 completely eliminated or significantly reduced their research and development
5 programs in the area of antibiotic research. According to a 1994 report by the
6 Rockefeller University Workshop in Multiple Antibiotic Resistant Bacteria, we
7 are on the verge of a "medical disaster that would return physicians back to the
8 pre-penicillin days when even small infections could turn lethal due to the lack
9 of effective drugs."

10 Despite recent advances in antimicrobial therapy and improved surgical
11 techniques, osteomyelitis (hard tissue or bone infection) is still a source of
12 morbidity often necessitating lengthy hospitalization. The failure of patients
13 with chronic osteomyelitis to respond uniformly to conventional treatment has
14 prompted the search for more effective treatment modalities. Local antibiotic
15 therapy with gentamicin-impregnated poly(methylmethacrylate) (PMMA) bead
16 chains (SEPTOPAL TM, E. Merck, West Germany) has been utilized in
17 Germany for the treatment of osteomyelitis for the past decade and has been
18 reported to be efficacious in several clinical studies. The beads are implanted
19 into the bone at the time of surgical intervention where they provide
20 significantly higher concentrations of gentamicin than could otherwise be
21 achieved via systemic administration. Serum gentamicin levels, on the other
22 hand, remain extremely low thereby significantly reducing the potential for
23 nephro- and ototoxicity that occurs in some patients receiving gentamicin
24 systemically.

-5-

1 Since SEPTOPAL TM is not currently approved by the Food and Drug
2 Administration for use in the United States, some orthopedic surgeons in this
3 country are fabricating their own "physician-made beads" for the treatment of
4 chronic osteomyelitis. A major disadvantage of the beads, however, is that
5 because the PMMA is not biodegradable it represents a foreign body and
6 should be removed at about 2-weeks postimplantation thereby necessitating in
7 some cases an additional surgical procedure. A biodegradable-biocompatible,
8 antibiotic carrier, on the other hand, would eliminate the need for this
9 additional surgical procedure and may potentially reduce both the duration as
10 well as the cost of hospitalization.

11 The concept of local, sustained release of antibiotics into infected bone
12 is described in recent literature wherein antibiotic-impregnated PMMA
13 macrobeads are used to treat chronic osteomyelitis. The technique as currently
14 used involves mixing gentamicin with methylmethacrylate bone cement and
15 molding the mixture into beads that are 7mm in diameter. These beads are
16 then locally implanted in the infected site at the time of surgical debridement to
17 serve as treatment. There are, however, significant problems with this
18 method. These include: 1) initially, large amounts of antibiotics diffuse from
19 the cement but with time the amount of antibiotic leaving the cement gradually
20 decreases to subtherapeutic levels; 2) the bioactivity of the antibiotic gradually
21 decreases; 3) methylmethacrylate has been shown to decrease the ability of
22 polymorphonuclear leukocytes to phagocytize and kill bacteria; 4) the beads do
23 not biodegrade and usually must be surgically removed; and 5) the exothermic
24 reaction that occurs during curing of methylmethacrylate limits the method to

-6-

1 the incorporation of only thermostable antibiotics (primarily aminoglycosides).
2 Nevertheless, preliminary clinical trials using these beads indicate that they are
3 equivalent in efficacy to longer term (4-6 weeks) administration of systemic
4 antibiotics.

5 In many instances, infectious agents have their first contact with the
6 host at a mucosal surface; therefore, mucosal protective immune mechanisms
7 are of primary importance in preventing these agents from colonizing or
8 penetrating the mucosal surface. Numerous studies have demonstrated that a
9 protective mucosal immune response can best be initiated by introduction of
10 the antigen at the mucosal surface, and parenteral immunization is not an
11 effective method to induce mucosal immunity. Antigen taken up by the gut-
12 associated lymphoid tissue (GALT), primarily by the Peyer's patches in mice,
13 stimulates T helper cell (Th) to assist in IgA B cell responses or stimulates T
14 suppressor cells (Ts) to mediate the unresponsiveness of oral tolerance.
15 Particulate antigen appears to shift the response towards the (Th) whereas
16 soluble antigens favor a response by the (Ts). Although studies have
17 demonstrated that oral immunization does induce an intestinal mucosal immune
18 response, large doses of antigen are usually required to achieve sufficient local
19 concentrations in the Peyer's patches. Unprotected protein antigens may be
20 degraded or may complex with secretory IgA in the intestinal lumen.

21 In the process of vaccination, medical science uses the body's innate
22 ability to protect itself against invading agents by immunizing the body with
23 antigens that will not cause the disease but will stimulate the formation of
24 antibodies that will protect against the disease. For example, dead organisms

-7-

1 are injected to protect against bacterial diseases such as typhoid fever and
2 whooping cough, toxins are injected to protect against viral diseases such as
3 poliomyelitis and measles.

4 It is not always possible, however, to stimulate antibody formation
5 merely by injecting the foreign agent. The vaccine preparation must be
6 immunogenic, that is, it must be able to induce an immune response. Certain
7 agents such as tetanus toxoid are innately immunogenic, and may be
8 administered in vaccines without modification. Other important agents are not
9 immunogenic, however, and must be converted into immunogenic molecules
10 before they can induce an immune response.

11 The immune response is a complex series of reactions that can
12 generally be described as follows:

- 13 1. the antigen enters the body and encounters antigen-presenting cells
14 which process the antigen and retain fragments of the antigen on their surfaces;
- 15 2. the antigen fragment retained on the antigen presenting cells are
16 recognized by T cells that provide help to B cells; and
- 17 3. the B cells are stimulated to proliferate and divide into antibody
18 forming cells that secrete antibody against the antigen.

19 Most antigens only elicit antibodies with assistance from the T cells
20 and, hence, are known as T-dependent (TD). These antigens, such as
21 proteins, can be processed by antigen presenting cells and thus activate T cells
22 in the process described above. Examples of such T-dependent antigens are
23 tetanus and diphtheria toxoids.

-8-

1 Some antigens, such as polysaccharides, cannot be properly processed
2 by antigen presenting cells and are not recognized by T cells. These antigens
3 do not require T cell assistance to elicit antibody formation but can activate B
4 cells directly and, hence, are known as T-independent antigens (TI). Such T-
5 independent antigens include *H. influenzae* type by polyribosyl-ribitol-phosphate
6 and pneumococcal capsular polysaccharides.

7 T-dependent antigens vary from T-independent antigens in a number of
8 ways. Most notably, the antigens vary in their need for an adjuvant, a
9 compound that will nonspecifically enhance the immune response. The vast
10 majority of soluble T-dependent antigens elicit only low level antibody
11 responses unless they are administered with an adjuvant. It is for this reason
12 that the standard DPT vaccine (diphtheria, pertussis, tetanus) is administered
13 with the adjuvant alum. Insolubilization of TD antigens into an aggregated
14 form can also enhance their immunogenicity, even in the absence of an
15 adjuvant. Golub ES and WO Weigle, J. Immunol. 102:389, 1969). In
16 contrast, T-independent antigens can stimulate antibody responses when
17 administered in the absence of an adjuvant, but the response is generally of
18 lower magnitude and shorter duration.

19 Four other differences between T-independent and T-dependent antigens
20 are:

21 a) T-dependent antigens can prime an immune response so that a
22 memory response can be elicited upon secondary challenge with the same
23 antigen. Memory or secondary responses are stimulated very rapidly and
24 attain significantly higher titers of antibody that are seen in primary responses.

1 T-independent antigens are unable to prime the immune system for secondary
2 responsiveness.

3 b) The affinity of the antibody for antigen increases with time
4 after immunization with T-dependent but not T-independent antigens.

5 c) T-dependent antigens stimulate an immature or neonatal
6 immune system more effectively than T-independent antigens.

7 d) T-dependent antigens usually stimulate IgM, IgG1, IgG2a, and
8 IgE antibodies, while T-independent antigens stimulate IgM, IgG1, IgG2b, and
9 IgG3 antibodies.

10 These characteristics of T-dependent vs. T-independent antigens provide
11 both distinct advantages and disadvantages in their use as effective vaccines.

12 T-dependent antigens can stimulate primary and secondary responses which are
13 long-lived in both adult and in neonatal immune systems, but must frequently
14 be administered with adjuvants. Thus, vaccines have been prepared using only
15 an antigen, such as diphtheria or tetanus toxoid, but such vaccines may require
16 the use of adjuvants, such as alum for stimulating optima responses.

17 Adjuvants are often associated with toxicity and have been shown to
18 nonspecifically stimulate the immune system; thus inducing antibodies of
19 specificities that may be undesirable.

20 Another disadvantage associated with T-dependent antigens is that very
21 small proteins such as peptides, are rarely immunogenic, even when
22 administered with adjuvants. This is especially unfortunate because many
23 synthetic peptides are available today that have been carefully synthesized to

-10-

1 represent the primary antigenic determinants of various pathogens, and would
2 otherwise make very specific and highly effective vaccines.

3 In contrast, T-independent antigens, such as polysaccharides, are able
4 to stimulate immune responses in the absence of adjuvants. Unfortunately,
5 however, such T-independent antigens cannot stimulate high level or prolonged
6 antibody responses. An even greater disadvantage is their inability to stimulate
7 an immature or B cell defective immune system (Mond J.J., Immunological
8 Reviews 64:99, 1982) Mosier DE, et al., J. Immunol. 119:1874, 1977).
9 Thus, the immune response to both T-independent and T-dependent antigens is
10 not satisfactory for many applications.

11 With respect to T-independent antigens, it is critical to provide
12 protective immunity against such antigens to children, especially against
13 polysaccharides such as *H. influenzae* and *S. pneumoniae*. With respect to T-
14 dependent antigens, it is critical to develop vaccines based on synthetic
15 peptides that represent the primary antigenic determinants of various
16 pathogens.

17 One approach to enhance the immune response to T-independent
18 antigens involves conjugating polysaccharides such *H. influenzae* PRP (Craze
19 J.M., Lewis R.E. Jr. ed., Conjugate vaccines in Contributions to Microbiology
20 and Immunology, vol. 10, 1989) or oligosaccharide antigens (Anderson PW, et
21 al., J. Immunol. 142:2464, 1989) to a single T-dependent antigen such as
22 tetanus or diphtheria toxoid. Recruitment of T cell help in this way has been
23 shown to provide enhanced immunity to many infants that have been
24 immunized. Unfortunately, only low level antibody titers are elicited, and

-11-

1 only some infants respond to initial immunizations. Thus, several
2 immunizations are required and protective immunity is often delayed for
3 months. Moreover, multiple visits to receive immunizations may also be
4 difficult for families that live distant from medical facilities (especially in
5 underdeveloped countries). Finally, babies less than 2 months of age may
6 mount little or no antibody response even after repeated immunization.

7 One possible approach to overcoming these problems is to
8 homogeneously disperse the antigen of interest within the polymeric matrix of
9 appropriately sized biodegradable-biocompatible microspheres that are
10 specifically taken up by GALT. Eldridge et al. have used a murine model to
11 show that orally-administered 1-10 micrometer microspheres consisting of
12 polymerized lactide and glycolide, (the same materials used in resorbable
13 sutures), were readily taken up into Peyer's patches, and the 1-5 micrometer
14 size were rapidly phagocytized by macrophages. Microspheres that were 5-10
15 micrometers (microns) remained in the Peyer's patch for up to 35 days,
16 whereas those less than 5 micrometer disseminated to the mesenteric lymph
17 node (MLN) and spleen within migrating MAC-1+ cells. Moreover, the
18 levels of specific serum and secretory antibody to staphylococcal enterotoxin B
19 toxoid and inactivated influenza A virus were enhanced and remained elevated
20 longer in animals which were immunized orally with microencapsulated
21 antigen as compared to animals which received equal doses of non-
22 encapsulated antigen. These data indicate that microencapsulation of an
23 antigen given orally may enhance the mucosal immune response against enteric
24 pathogens. AF/R1 pili mediate the species-specific binding of *E. coli* RDEC-1

-12-

1 with mucosal glycoproteins in the small intestine of rabbits and are therefore
2 an important virulence factor. Although AF/R1 pili are not essential for E.
3 coli RDEC-1 to produce enteropathogenic disease, expression of AF/R1 to
4 produce enteropathogenic disease, expression of AF/R1 promotes a more
5 severe disease. Anti-AF/R1 antibodies have been shown to inhibit the
6 attachment of RDEC-1 to the intestinal mucosa and prevent RDEC-1 disease in
7 rabbits. The amino acid sequence of the AF/R1 pilin subunit has recently been
8 determined, but specific antigenic determinants within AF/R1 have not been
9 identified.

10 In the current study we have used these theoretical criteria to predict
11 probable T or B cell epitopes from the amino acid sequence of AF/R1. Four
12 different 16 amino acid peptides that include the predicted epitopes have been
13 synthesized: AF/R1 40-55 as a B cell epitope, 79-94 as a T cell epitope, 108-
14 123 as a T and B cell epitope, and AF/R1 40-47/79-86 as a hybrid of the first
15 eight amino acids from the predicted B cell epitope and the T cell epitope. We
16 have used these peptides as well as the native protein to stimulate the *in vitro*
17 proliferation of lymphocytes taken from the Peyer's patch, MLN, and spleen
18 of rabbits which have received intraduodenal priming with microencapsulated
19 or non-encapsulated AF/R1. Our results demonstrate the microencapsulation
20 of AF/R1 potentiates the cellular immune response at the level of the Peyer's
21 patch, thus enhancing *in vitro* lymphocyte proliferation to both the native
22 protein and its linear peptide antigens. CFA/I pili, rigid thread-like structures
23 which are composed of repeating pilin subunits of 147 amino acid found on
24 serogroups 015, 025, 078, and 0128 of enterotoxigenic E. coli (ETEC) (1-4,

-13-

1 18). CFA/I promotes mannose resistant attachment to human brush borders
2 (5); therefore, a vaccine that established immunity against this protein may
3 prevent the attachment to host tissues and subsequent disease. In addition,
4 because the CFA/I subunit shares N-terminal amino acid sequence homology
5 with CS1, CFA/II (CS2) and CFA/IV (CS4) (4), a subunit vaccine which
6 contained epitopes from this area of the molecule may protect against infection
7 with various ETEC.

8 Until recently, experiments to identify these epitopes were time
9 consuming and costly; however, technology is now available which allows one
10 to simultaneously identify all the T cell and B cell epitopes in the protein of
11 interest. Multiple Peptide synthesis (Pepscan) is a technique for the
12 simultaneous synthesis of hundreds of peptides on polyethylene rods (6). We
13 have used this method to synthesize all the 140 possible overlapping
14 actapeptides of the CFA/I protein. The peptides, still on the rods, can be used
15 directly in ELISA assays to map B cell epitopes (6, 12-14). We have also
16 synthesized all the 138 possible overlapping decapeptides of the CFA/I protein.
17 For analysis of T cell epitopes, these peptides can be cleaved from the rods
18 and used in proliferation assays (15). Thus this technology allows efficient
19 mapping and localization of both B cell and T cell epitopes to a resolution of a
20 single amino acid (16). These studies were designed to identify antigenic
21 epitopes of ETEC which may be employed in the construction of an effective
22 subunit vaccine.

23 CFA/I pili consist of repeating pilin protein subunits found on several
24 serogroups of enterotoxigenic E coli (ETEC) which promote attachment to

-14-

1 human intestinal mucosa. We wished to identify areas within the CFA/I
2 molecule that contain immunodominant T cell epitopes that are capable of
3 stimulating the cell-mediated portion of the immune response in primates as
4 well as immunodominant B cell epitopes. To do this, we (a) resolved the
5 discrepancy in the literature on the complete amino acid sequence of CFA/I,
6 (b) immunized three Rhesus monkeys with multiple i.m. injections of purified
7 CFA/I subunit in Freund's adjuvant, (c) synthesized 138 overlapping
8 decapeptides which represented the entire CFA/I protein using the Pepscan
9 technique (Cambridge Research Biochemicals), (d) tested each of the peptides
10 for their ability to stimulate the spleen cells from the immunized monkeys in a
11 proliferative assay (e) synthesized 140 overlapping octapeptides which
12 represented the entire CFA/I protein, and (f) tested serum from each monkey
13 for its ability to recognize the octapeptides in a modified ELISA assay. A total
14 of 39 different CFA/I decapeptides supported a significant proliferative
15 response with the majority of the responses occurring within distinct regions of
16 the protein (peptides beginning with residues 8-40, 70-80, and 126-137).
17 Nineteen of the responsive peptides contained a serine residue at positions 2,
18 3, or 4 in the peptide, and a nine contained a serine specifically at position 3.
19 Most were predicted to be configured as an alpha helix and have a high
20 amphipathic index. Eight B cell epitopes were identified at positions 3-11, 11-
21 21, 22-29, 32-40, 38-45, 66-74, 93-101, and 124-136. The epitope at position
22 11-21 was strongly recognized by all three individual monkeys, while the
23 epitopes at 93-101, 124-136, 66-74, and 22-29 were recognized by two of the
24 three monkeys.

-15-

1 Recent advances in the understanding of B cell and T cell epitopes have
2 improved the ability to select probably linear epitopes from the amino acid
3 sequence using theoretical criteria. B cell epitopes are often composed of a
4 string of hydrophilic amino acids with a high flexibility index and a high
5 probability of turns within the peptide structure. Prediction of T cell epitopes
6 are based on the Rothbard method which identifies common sequence patterns
7 that are common to known T cell epitopes or the method of Berzofsky and
8 others which uses a correlation between algorithms predicting amphipathic
9 helices and T cell epitopes.

10 V. SUMMARY OF THE INVENTION

11 This invention relates to active core materials such as biologically
12 active agent(s), drug(s), or substance(s) encapsulated within a biodegradable-
13 biocompatible polymeric matrix. In view of the enormous scope of this
14 invention it will be presented herein as Phases I, II, and III. Phase I illustrates
15 the encapsulation of antibiotics within a biodegradable-biocompatible
16 polymeric matrix for the prevention and treatment of wound infections. Phase
17 II illustrates the encapsulation of antigens ^{including tumor antigens and} (more specifically, oral-intestinal
18 vaccine antigens) within a biodegradable-biocompatible polymeric matrix
19 against diseases such as those caused by enteropathogenic organism. Phase III
20 illustrates the use of a biodegradable-biocompatible polymeric matrix for burst-
21 free programmable sustained release of biologically active agents, inclusive of
22 peptides, over a period of up to 100 days in an aqueous physiological
23 environment.

-16-

1 Controlled drug delivery from a biodegradable-biocompatible matrix
2 offers profound advantages over conventional drug/antigen dosing.
3 Drugs/antigens can be used more effectively and efficiently, less drug/antigen
4 is required for optimal therapeutic effect and, in the case of drugs, toxic side
5 effects can be significantly, reduced or essentially eliminated through drug
6 targeting. The stability of some drugs/antigens can be improved allowing for a
7 longer shelf-life, and drugs/antigens with a short half-life can be protected
8 within the matrix from destruction, thereby ensuring sustained release of active
9 agent over time. The benefit of a continuous sustained release of drug/antigen
10 is beneficial because drug levels can be maintained within a constant
11 therapeutic range and antigen can be presented either continuously or in a
12 pulsatile mode as required to stimulate the optimal immune response. All of
13 this can be accomplished with a single dose of encapsulated drug/antigen.

14 This invention contemplates, but is not limited to, medically acceptable
15 methods for the effective local delivery of biologically active agents that, of
16 themselves, are directly (e.g. drugs, such as antibiotics) or indirectly (e.g.
17 vaccine antigens) therapeutic or prophylactic. It also includes drugs/agents that
18 elicit/modulate natural biological activity.

19 Wounds characterized by the presence of infection, devitalized tissue,
20 and foreign-body contaminants have high infection rates and are difficult to
21 treat. This invention describes antibiotic formulation encapsulated within
22 microspheres of a biodegradable-biocompatible polymer that, when applied
23 locally to contaminated or infected wounds, provides immediate, direct, and
24 sustained (over a period up to 100 days), high concentrations of antibiotic in

-17-

1 the wound site (soft tissue and bone). By encapsulating antibiotics and
2 applying them directly, one can achieve a significant reduction in nonspecific
3 binding of the drug to body proteins, a phenomena commonly observed
4 following conventional systemic administration of free drugs. Thus, less drug
5 is required, higher concentrations are maintained at the site of need, and
6 efficacy is enhanced. This approach provides superior treatment over
7 conventional systemic administration of antibiotics for wound infections
8 because higher bacteriocidal concentrations can be achieved and maintained in
9 the wound environment. Higher concentrations kill more bacteria.
10 Applicants' invention for this application is described in Phase I.
11 Furthermore, applicants reasoned that a protective mucosal immune response
12 might be best initiated by introduction of an antigen at the mucosal surface,
13 because unprotected protein antigens delivered in a free form may be degraded
14 or may complex with secretory IgA in the intestinal lumen precluding entry
15 and subsequent processing in local immune cells. The formulation of
16 microspheres containing antigen small enough in size to be phagocytized
17 locally in the gut was envisioned as being able to induce an elevated localized
18 immune response. Applicants' invention for this application is described in
19 Phase II. In summary, applicants propose using several methods for the local
20 application of drugs including: 1) the direct application of the encapsulated
21 drug to a surgical/traumatized area, 2) oral delivery that provides either local
22 deposition of microencapsulated antigen/drugs at mucosal membranes or
23 transport across these membranes to provide local adherence of
24 microencapsulated drugs/antigen to mucosal membranes to provide sustained

-18-

1 release of drug/antigen into soft tissue or a body cavity, and/or 3) sustained
2 intercellular or extracellular drug/antigen release following subcutaneous
3 injection.

4 In those instances where antibiotics are administered locally, applicants
5 have found that the controlled release of the antibiotic from within a
6 biodegradable-biocompatible polymeric matrix within 14 days to about 4
7 weeks without significant drug trailing is especially useful. However, if
8 desired, the release of a biologically active agent from a polymeric matrix
9 comprised of an active agent and a blend of uncapped and end-capped
10 biodegradable poly DL(lactide-co-glycolide), can be controlled over a period of
11 1 to about 100 days without significant drug dumping or trailing. Such novel
12 biocompatible-biodegradable microspheres developed with a burst-free
13 programmable sustained release of biologically active agents, inclusive of
14 polypeptides, are described in applicants' U.S. Patent Application Serial No.
15 08/590,973 filed January 24, 1996.

16 When antibiotics are administered systemically in the conventional
17 manner, or locally as contemplated by the applicants, the immune response to
18 the antibiotic and the potential for hypersensitivity and/or anaphylactoid
19 response (especially to beta-lactam antibiotics such as penicillins/ampicillin) is
20 a clinical concern. In early studies the inventors observed a specific IgG
21 response to ampicillin as it was released from the microencapsulated
22 formulation (illustrated in the histogram, Figure 1 and 2). This response is
23 reminiscent of antibody elicited by vaccine antigens in conventional vaccines.
24 The response to vaccine antigens is known to be accentuated by the use of an

-19-

1 adjuvant such as alum. Alum is a crude, less adaptable delivery vehicle than
2 its counterpart, the biodegradable-biocompatible poly DL(lactide-co-glycolide),
3 of this invention - the polymeric matrix. This knowledge stimulated additional
4 studies relevant to the effects of sustain release of agents on the immune
5 response.

6 There are, in general, two forms of localized delivery which can be
7 achieved with PLGA microspheres-delivery which is localized to individual
8 cells of the body (intracellular delivery); and delivery which is localized to
9 tissues within a specific region of the body (localized extracellular delivery).

10 Applicants have prepared antibiotic and hepatitis vaccine formulations
11 which functioned by delivering localized extracellular doses of their active
12 agents. This was achieved by using relatively large microspheres which served
13 as a depot for the drug or antigen. Their large size 40-100 microns in diameter
14 precluded their being phagocytized or diffusing throughout the intercellular
15 fluid compartments of the body. Their drug agent loads were thus released
16 within their immediate vicinity which resulted in the generation of very high
17 local concentrations of antibiotic or the release of sufficiently high
18 concentrations of free antigen to induce an immune response.

19 The large-diameter antibiotic bearing microspheres were originally
20 developed by applicants primarily for topical application on exposed debrided
21 tissues of combat wounds. However, an inherent property exhibited by the
22 antibiotics when topically applied to a wound site is the generation of
 measurable levels of immune response. This concept of local delivery by

-20-

1 topical application of microspheres to tissue to achieve localized concentrations
2 of therapeutic agents was subsequently applied to the development of an oral
3 vaccine for protection against traveler's diarrhea caused by E. coli. Vaccine
4 antigen was encapsulated into microspheres whose diameters were
5 predominantly in the 5-10 micron size range based on an understanding that
6 microspheres of this size would not readily be either phagocytized or
7 transported across the gut wall into the body. Ingestion of these microspheres
8 thus constituted a localized delivery achieved by topical application of the
9 spheres to the wall tissue of the gut. This topical application resulted in the
10 localized trapping of a small percentage of these sphere into the Peyer's
11 patches where the spheres proceeded to release their antigen in a localized
12 fashion to immune cells located within the intestinal Patches.

13 The concept of localized sustained local delivery has been further
14 extended to the delivery of analgesics and anesthetics to exposed dental pulp to
15 control pain and inflammatory responses. Again, the PLGA microsphere used
16 for this type of delivery are relatively large (40-100 um in diameter) and serve
17 as a topical depot for localized extracellular release of the drug.

18 Consistent with their understanding of the inherent immunogenic
19 properties exhibited by active core materials *in vivo*, applicants have moved on
20 to other non-topical application methods of using their microsphere delivery
21 system. Some of these center on the use of small diameter microspheres
22 ranging from sub micron to under 5 microns in diameter. These spheres allow
23 intracellular targeting of drug or antigen. They also allow for transmucosal
24 delivery of drugs or antigens. The concept of localized delivery in these

-21-

1 instances refers to the localized delivery of drug or agent within individual
2 target cells of the body regardless of their location or distribution within the
3 body. This approach is useful in development of antitubercular, antimalarial,
4 antiviral, and antichlamydial formulations against intracellular parasites. It is
5 also useful for the development of vaccines against intracellular parasites and
6 for direct delivery of agents to presenting cells of the immune system.

7 Another nontopical application method of using PLGA microspheres
8 resides in their usefulness as injectable depots for drugs intended for either
9 localized or systemic delivery. Typically larger diameter microspheres are
10 used for depots as these are less likely to diffuse away. The local or systemic
11 nature of these delivery systems is, in part a function of the release rate of the
12 drug from the depot and the diffusional and solubility characteristics of the
13 drug being released. Cancer chemotherapeutics, systemic antibiotics, delivery
14 of antibiotics to infected bone are potential application of this system.

15 Additional this non-topical systemic depot application can be extended to the iv
16 injection of cancer-agent laden microspheres to embolize and destroy a
17 malignant tumor. Additionally, the PLGA microspheres can be used as a
18 carrier to deliver substances useful for the in modification of cells or genes in
19 bioengineering or genetic procedures.

20 Interest in the concept that antigens encapsulated within a
21 biodegradable-biocompatible polymeric matrix could be formulated as a
22 vaccine with superior efficacy over conventional vaccines, originated from the
23 inventors' own observations that the drug, ampicillin, when sustain released
24 from poly DL(lactide-co-glycolide) elicited antibody production. In these

-22-

1 studies, the applicants were able to measure specific IgG antibodies to free
2 ampicillin and to ampicillin released from microencapsulated ampicillin
3 formulations in the sera of mice previously "treated" with the ampicillin
4 formulations using ELISA. Numerous other studies also document the ability
5 of beta-lactam antibiotic to elicit antibody. Selected, more recent studies
6 whose findings are consistent with earlier discoveries made by applicants when
7 conducting experiments with ampicillin include those by Klein et al. (1993)
8 who detected specific IgG antibodies (IgG and IgG3 subclasses) to the B-
9 lactam ring in patients receiving penicillin therapy, work by Nagakura et al.
10 (1990) which detected specific antibodies to cephalexin, a B-lactam antibiotic
11 in the sera of guinea pigs, and Auci et al. (1993) who detected benzyl
12 penicilloyl specific IgM, IgG IgE, and IgA antibody forming cells in lymphoid
13 cells of mice given benzyl penicilloyl-Keyhole Limpet Hemocyanin.
14 Pharmaceutical compositions of antigens encapsulated with poly DL(lactide-co-
15 glycolide) are described in Phase II. The microspheres of the invention allow
16 for introduction of vaccine antigens to mucosal surfaces in particles that can be
17 subsequently taken up locally by phagocytic cells. Such an approach for both
18 drugs and antigens provides significant advantages in potency and efficacy over
19 conventional systemically administered drugs or vaccines. A partial list of
20 biologically active agents or drugs that will potentially derive significant
21 medical benefits from this delivery system includes: antibacterial agents;
22 peptides; polypeptides; antibacterial peptides; antimycobacterial agents;
23 antimycotic agents; antiviral agents; antiparasitic agents; antifungal; antiyeast
24 agents; hormonal peptides; cardiovascular agents; hormonal

-23-

1 peptides; cardiovascular agents; narcotic antagonists; analgesics; anesthetics;
2 insulins; steroids including HIV therapeutic drugs (including protease
3 inhibitors) and AZT; estrogens; progestins; gastrointestinal therapeutic agents;
4 non-steroidal anti-inflammatory agents; parasympathomimetic agents;
5 psychotherapeutic agents; tranquilizers; decongestants; sedative-hypnotics; non-
6 estrogenic and non-progestional steroids; sympathomimetic agents; vaccines;
7 vitamins; nutrients; anti-migraine drugs; electrolyte replacements; ergot
8 alkaloids; anti-inflammatory agents; prostaglandins; cytotoxic drugs; antigens;
9 antibodies; enzymes; growth factors; immunomodulators; pheromones;
10 prodrugs; psychotropic drugs; nicotine; antiblood clotting drugs; appetite
11 suppressants/stimulants and combinations thereof; contraceptive agents include
12 estrogens such as diethyl silbestrol; 17-beta-estradiol; estrone; ethinyl estradiol;
13 mestranol; progestins such as norethindrone; norgestryl; ethynodiol diacetate;
14 lynestrenol; medroxyprogesterone acetate; dimethisterone; megestrol acetate;
15 chlormadinone acetate; norgestimate; norethisterone; ethisterone; melenlate;
16 norgestimate; norethisterone; ethisterone; melengestrol; norethynodrel; and
17 spermicidal compounds such as nonyphenoxypolyoxyethylene glycol;
18 benzethonium chloride; chlorindanol; include gastrointestinal therapeutic agents
19 such as aluminum hydroxide; calcium carbonate; magnesium carbonate;
20 sodium carbonate and the like; non-steroidal antifertility agents;
21 parasympathomimetic agents; psychotherapeutic agents; major tranquilizers
22 such as chloropromazine HCl; clozapine; mesoridazine; metiapine;
23 reserpine; thioridazine; minor tranquilizers such as chlordiazepoxide;
24 diazepam; meprobamate; temazepam and the like; rhinological decongestants;

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☒ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.